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Synthesis and biological properties of chemically modified siRNAs bearing 1-deoxy-D-ribofuranose in their 3′-overhang region

Kazumi Taniho [a], Remi Nakashima [b], Mahmoud Kandeel [b], Yoshiaki Kitamura [a], Yukio Kitade [a,b,*]

[a] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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ABSTRACT

To elucidate the role of the sugar moiety in the two natural nucleotides of the 3′-overhang region of small interfering RNA (siRNA), we synthesized siRNAs that incorporated two abasic nucleosides, 1-deoxy-D-ribofuranose (R^H). We improved the method for preparing an *O*-protected abasic nucleoside, 1-deoxy-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, via the reductive cleavage of the anomeric position of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose. To incorporate R^H into oligonucleotides by the standard phosphoramidite solid phase method, R^H was converted into its phosphoramidite derivative and the solid support linked to a controlled pore glass resin. Chemically modified RNAs possessing R^H at the 3′-overhang region were easily prepared in good yields. siRNAs containing R^H showed moderate nuclease-resistance and a desirable knockdown effect.

Chemically modified nucleosides have been utilized as biologically important compounds, for example, in virus therapy, cancer therapy, and molecular biology.[1,2] Furthermore, functional oligonucleotides (ONs) containing artificial nucleic acids have been employed in diagnostic and therapeutic applications.[3,4] Because nucleic acid medicines such as antisense ONs and small interfering and microRNAs (siRNAs and miRNAs, respectively) can be used for sequence-dependent gene silencing,[4] the development of nucleic acid medicines has attracted considerable attention from medicinal chemists.

We recently reported the synthesis and biological properties of modified siRNAs[5] and miRNAs[6] bearing hydrophobic 1,3-bis(hydroxymethyl)benzene (B) and/or 1,3-bis(hydroxymethyl)-pyridine (P) in their 3′-overhang regions (Fig. 1). The 3′-overhang region of an antisense strand or guide strand in the case of miRNAs is recognized by the Piwi/Argonaute/Zwille (PAZ) domain of Ago2, and the 2 nucleotides at the 3′-end are accommodated into the binding pocket of the PAZ domain, which is composed of hydrophobic amino acids.[7] The silencing effect of BB-modified siRNAs was almost equal to that of normal siRNAs containing natural nucleotides in the 3′-overhang region.[5] Furthermore, the analogously BP-modified miRNA (miR-143/BP) showed a significant tumor-suppressive effect on xenografted tumors of DLD-1 human cancer cells.[6]

1-Deoxy-D-ribofuranose (R^H; **1**), an abasic nucleoside, is a useful analog for studying the structures and mechanisms of action of bioactive ONs. Several modified ONs containing R^H have been designed and synthesized.[8] Several reports have described the stereoselective synthesis of R^H;[9] however, most of the existing protocols require multiple steps. Zhou and co-workers succeeded in the direct conversion of D-ribose into R^H, but the reaction generated a moderate yield.[10]

To elucidate the role of the sugar moiety at the two natural nucleotides of the 3′-overhang region, we synthesized siRNA incorporating an abasic nucleoside (R^H) dimer at this position of each RNA sequence (Fig. 2). We evaluated their binding activity to the PAZ domain and knockdown effect. Furthermore, we improved the synthetic method for preparing R^H.

Scheme 1 shows a convenient method for **1** by using commercially available 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**) as a starting material. Treatment of **2** with trimethylsilyl trifluoromethanesulfonate (TMSOTf) and triethylsilane (Et_3SiH) in MeCN at room temperature[11] gave the desired deoxygenated product **3** in quantitative yield. Deprotection of the benzoyl groups of **3** followed by benzylation gave **4**. Removal of all benzyl groups by catalytic hydrogenation afforded **1** (94% overall yield from **2**).

To incorporate **1** into an ON by the standard phosphoramidite solid phase method, **4** was converted into phosphoramidite derivative **8** and solid support **9** linked to controlled pore glass (CPG) resin. Treatment of **1** with 4,4′-dimethoxytrityl (DMTr) chloride in a mixture of DMF and pyridine gave the corresponding 5-DMTr derivative **5** in 75% yield. Subsequent silylation gave the corresponding 2-protected isomer **6** and 3-protected isomer **7** in yields of 32% and 30%, respectively. The 2-protected isomer **6** was phosphitylated by standard procedure to produce the

* Corresponding author. Tel./fax: +81 58 293 2640.
E-mail address: ykkitade@gifu-u.ac.jp (Y. Kitade).

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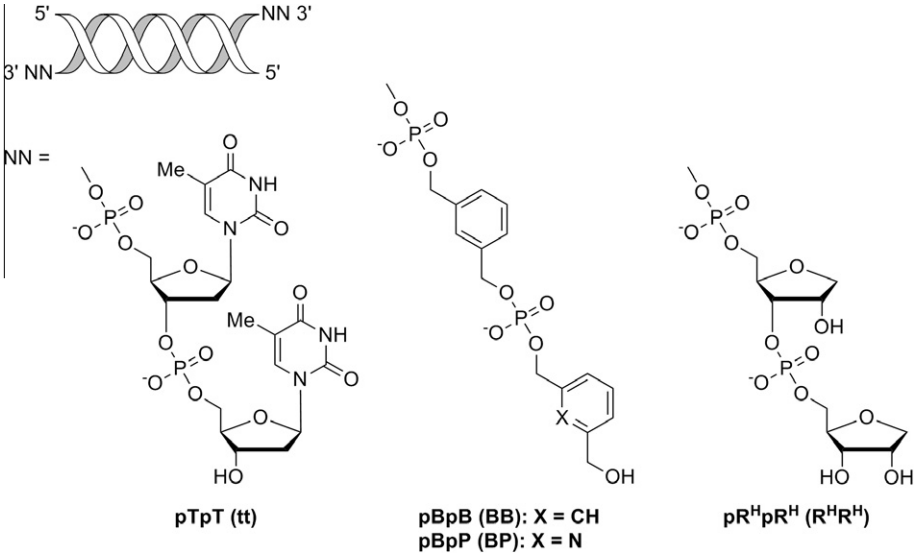


Figure 1. Structures of the modified siRNAs.

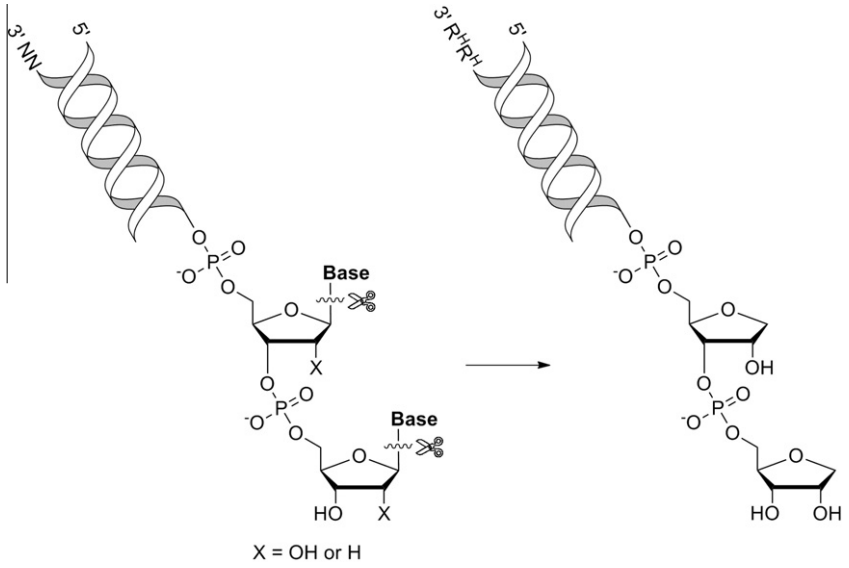


Figure 2. Concept for this study.

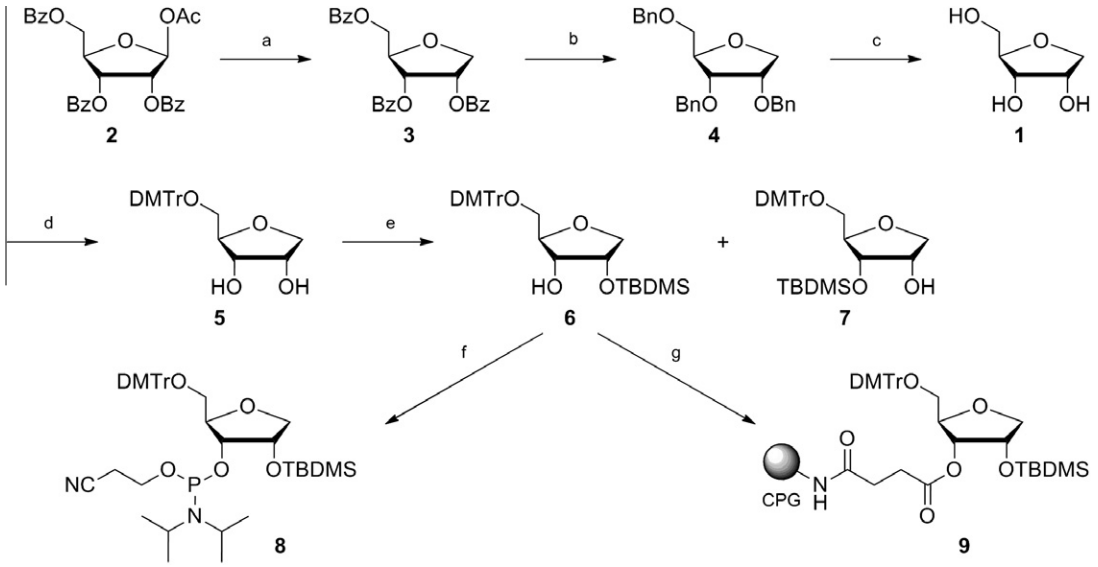
corresponding phosphoramidite **8**, quantitatively. On the other hand, **6** was succinated to yield the corresponding succinate, which was linked to CPG to generate solid support **9** (42 μmol/g).

Oligoribonucleotide synthesis: By using **8** and **9**, ONs containing R^H were synthesized with a DNA/RNA synthesizer (Table 1). The fully protected ONs linked to a solid support were treated with concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h and with tetra-*n*-butylammonium fluoride (TBAF; 1.0 M solution in THF) at room temperature for 12 h. The ONs were purified by denaturing 20% polyacrylamide gel electrophoresis (PAGE) to isolate deprotected ONs bearing the abasic nucleoside R^H. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights were in good agreement with their structures.[12]

Thermal stability: [13] siRNA thermal stability was studied by thermal denaturation in a buffer of 10 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. Table 2 shows the melting temperatures (T_ms) of the siRNAs. The T_m value of siRNA **11** (T_m = 77.9 °C), which had R^H in the 3′-overhang region, was almost equal to that of the unmodified siRNA **10** (T_m = 79.7 °C), which had thymidine in the overhang region. Thus, it was found that the deletion of a nucleobase did not have a crucial effect on the thermal stability of the duplex.

Dual-luciferase assay: [14] We examined the silencing activity of siRNAs **10** and **11** by a dual-reporter assay using a psiCHECK-2 vector in HeLa cells. The vector contains the *Renilla* and firefly luciferase genes, and the siRNA sequences were designed to target the *Renilla* luciferase gene. HeLa cells were co-transfected with the vector and the indicated amount of siRNAs. The levels of *Renilla* luciferase were normalized to those of firefly luciferase. The silencing activity of the siRNA with the abasic nucleosides was found to be greater than that of siRNA **10**, which possessed the natural nucleosides in the 3′-overhang region (Fig. 3).

Nuclease resistance: [15] Improving the nuclease resistance of synthetic siRNAs is important for their therapeutic application. It was



Scheme 1. Reagents and conditions: (a) Et_3SiH, TMSOTf, MeCN, rt, quant; (b) (i) NaH, MeOH, rt; (ii) BnBr, NaH, DMF, rt, quant; (c) H_2, $Pd(OH)_2/C$, MeOH, rt, 94%; (d) DMTrCl, DMF, pyridine, rt, 89%; (e) TBDMSCl, imidazole, DMF, rt, 32% for **6** and 31% for **7**; (f) $(i\text{-}Pr_2N)P(Cl)O(CH_2)_2CN$, $i\text{-}Pr_2NEt$, THF, rt, 91%; (g) (i) succinic anhydride, DMAP, pyridine, rt; (ii) CPG, EDCI, DMF, rt, 42 μmol/g.

Table 1
Sequences of ONs and siRNAs used in this study

No.of siRNA	No. of ON	Sequence
siRNA **10**	ON **12**	5′-GGCCUUUCACUACUCCUACtt-3′
	ON **13**	3′-ttCCGGAAAGUGAUGAGGAUG-5′
siRNA **11**	ON **14**	5′-GGCCUUUCACUACUCCUACR^HR^H-3′
	ON **15**	3′-R^HR^HCCGGAAAGUGAUGAGGAUG-5′
–	ON **16**	F-5′-GUAGGAGUAGUGAAAGGCCtt-3′
–	ON **17**	F-5′-GUAGGAGUAGUGAAAGGCCR^HR^H-3′

[a]Capital letters indicate ribonucleosides and small italicized letters show 2′-deoxyribonucleosides. [b]F denotes fluorescein.

Table 2
Melting temperatures (T_ms) of siRNAs

No.of siRNA	T_m (°C)
siRNA **10**	79.7
siRNA **11**	77.9

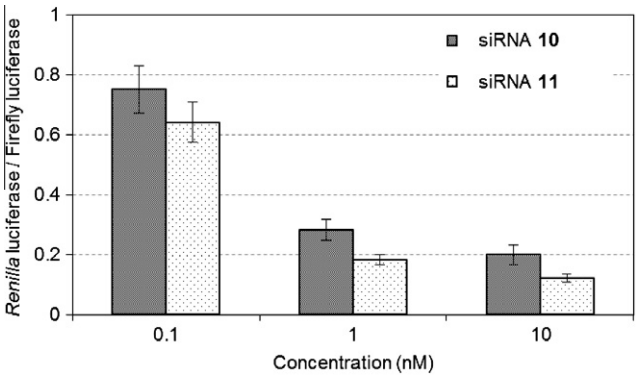


Figure 3. Dual-luciferase assay.

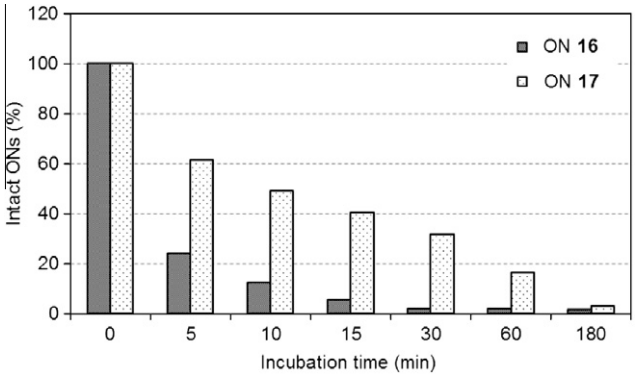


Figure 4. Nuclease resistance of ON **16** and ON **17** against SVPD.

expected that abasic nucleoside-substituted RNA would be more resistant to nucleases than unmodified RNAs. The susceptibility of RNAs to snake venom phosphodiesterase (SVPD), a 3′-exonuclease, was examined. Unmodified ON **16** and modified ON **17**, which were labeled at their 5′-ends with fluorescein, were incubated with SVPD. The reactions were analyzed using PAGE under denaturing conditions. Unmodified ON **16** was hydrolyzed after 5 min of incubation, while modified ON **17** was resistant to the enzyme (Fig. 4). The half-life ($t_{1/2}$) of unmodified ON **16** was <5 min, and that of modified ON **17** was 10 min. ON **17** carrying an R^H dimer at its 3′-end was significantly more resistant to SVPD than unmodified ON **16**.

In conclusion, we demonstrated a practical method for the preparation of R^H and the synthesis of siRNAs containing R^H in their 3′-overhang region. The silencing activity of the siRNAs was examined by using the dual-luciferase assay. It was found that the siRNA with the abasic nucleosides in the 3′-overhang region was more effective than the siRNA possessing the natural nucleosides in this region in an in vitro dual-luciferase experiment. Furthermore, the modified RNA possessing R^H was more resistant to nucleolytic hydrolysis by SVPD than the unmodified RNA. Thus, the R^H modification may hold promise as a method to improve the silencing activity and nuclease-resistance of RNA medicines.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.132.

References and notes

1. For a review on modern trends in biotechnology and application of natural and modified nucleosides, see: (a) Mikhailopulo, I. A.; Miroshnikov, A. I. *Acta Naturae* **2010**, *2*, 36; (b) Mikhailopulo, I. A.; Miroshnikov, A. I. *Mendeleev Commun.* **2011**, *21*, 57.
2. For a review on synthetic strategies and biological applications of C-nucleosides, see: Stambasky, J.; Hocek, M.; Kocovsky, P. *Chem. Rev.* **2009**, *109*, 6729.
3. For a review on oligonucleotides as antivirals, see: Mescalchin, A.; Restle, T. *Molecules* **2011**, *16*, 1271.
4. For a review on oligonucleotides as RNAi-based therapy, see (a) Czech, M. P.; Aouadi, M.; Tesz, G. J. *Nat. Rev. Endocrinol.* **2011**, *7*, 473; (b) Lennox, K. A.; Behlke, M. A. *Gene Therapy* **2011**, *18*, 1111.
5. Ueno, Y.; Watanabe, Y.; Shibata, A.; Yoshikawa, K.; Takano, T.; Kohara, M.; Kitade, Y. *Bioorg. Med. Chem.* **2009**, *17*, 1974.
6. (a) Akao, Y.; Nakagawa, Y.; Hirata, I.; Iio, A.; Itoh, T.; Kojima, K.; Nakashima, R.; Kitade, Y.; Naoe, T. *Cancer Gene Ther.* **2010**, *17*, 398; (b) Kitade, Y.; Akao, Y. J. *Pharmacol. Sci.* **2010**, *114*, 276; (c) Noguchi, S.; Mori, T.; Hoshino, Y.; Maruo, K.; Yamada, N.; Kitade, Y.; Naoe, T.; Akao, Y. *Cancer Lett.* **2011**, *307*, 211.
7. (a) Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. *Nature* **2003**, *426*, 465; (b) Yan, K. S.; Yan, S.; Farooq, A.; Han, A.; Zeng, L.; Zhou, M. M. *Nature* **2003**, *426*, 469; (c) Song, J. J.; Liu, J.; Tolia, N. H.; Schneiderman, J.; Smith, S. K.; Martienssen, R. A.; Hannon, G. J.; Joshua-Tor, L. *Nat. Struct. Biol.* **2003**, *12*, 1026; (d) Ma, J. B.; Te, K.; Patel, D. J. *Nature* **2004**, *429*, 318.
8. (a) Schmidt, S.; Beigelman, L.; Karpeisky, A.; Usman, N.; Sørensen, U. S.; Gait, M. J. *Nucleic Acids Res.* **1996**, *24*, 573; (b) Efimov, V. A.; Buryakova, A. A.; Chakhmakhcheva, O. G. *Nucleic Acids Res.* **1999**, *27*, 4416; (c) Kim, H. B.; Kim, S. Y.; Gil, J. M.; Park, H. J.; Park, H. O. WO Patent 045067, **2009**.; (d) Butora, G.; Davies, I. W.; Flana-Gan, F. M.; Fu, W.; Kenski, D. M.; Di, N. WO Patent 084345, **2011**.
9. (a) Beigelman, L.; Karpeisky, A.; Usman, N. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1715; (b) Hossain, N.; van Halbeek, H.; De Clercq, E.; Herdewijn, P. *Tetrahedron* **1998**, *54*, 2209.
10. Alfaro, J. F.; Zhang, T.; Wynn, D. P.; Karschner, E. L.; Zhou, Z. S. *Org. Lett.* **2004**, *6*, 3043.
11. Jeffery, A.; Nair, V. *Tetrahedron Lett.* **1995**, *36*, 3627.
12. *MALDI-TOF/MS analyses of ONs*. The following spectra were obtained by MALDI-TOF/MS (negative mode). ON 14: calculated mass, 6282.8; observed mass, 6282.9. ON 15: calculated mass, 6591.9; observed mass, 6595.3. ON 17: calculated mass, 7130.1; observed mass, 7128.1.
13. *Thermal denaturation study*. Each solution containing each siRNA (3.0 μM) in a buffer composed of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) and 100 mM NaCl was heated at 95 °C for 3 min, then cooled gradually to an appropriate temperature, and used for the thermal denaturation studies. Thermal-induced transitions of each mixture were monitored at 260 nm with a spectrophotometer.
14. *Dual-luciferase assay*. HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in D-MEM (Wako) supplemented with 10% bovine serum (Sigma). 24 h before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL per well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psi-CHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. Transfection without siRNA was used as a control. After 1 h, D-MEM (100 μL) containing 10% bovine serum was added to each well, and the whole was further incubated at 37 °C. After 24 h, cell extracts were frozen at −80 °C. Activities of firefly and Renilla luciferases in cell lysates were determined with a Dual-Glo Luciferase Assay System (Promega). The results were confirmed by at least three independent transfection experiments with four cultures each and are expressed as the average from three experiments as mean ± SD.
15. *Partial hydrolysis of ONs with snake venom phosphodiesterase*. Each ON (300 pmol) labeled with fluorescein at the 5′-end was incubated with snake venom phosphodiesterase (3 ng) in a buffer containing 37.5 mM Tris–HCl (pH 7.0) and 7.5 mM $MgCl_2$ (total 100 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9.0 M urea (15 μL). The mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 7.0 M urea. The labeled ON in the gel was visualized by a lumino image analyzer.